High throughput cell-based screening strategies for inflammatory receptors

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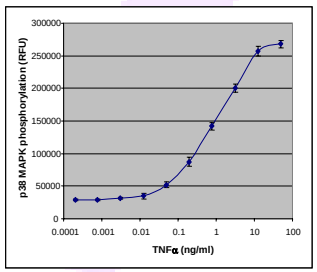
INTRODUCTION

Inflammatory mediators, such as TNFα, activate specific cellular pathways on select cell types to exert their biological effects. Pharmaceutical screening of inhibitors of the action of these mediators, acting either at the cell surface receptor or at intracellular signal transduction targets, requires assays utilizing intact cells. Only cellular systems can provide the biological information required of such screens, including inactivation of receptor function (rather than just compound binding), cytosolic access for intracellular inhibitors, and specificity against other signal transduction pathways and/or receptors.
Key signaling molecules that mediate inflammatory action in several cellular and animals systems are p38 MAP kinase, JNK, Stat-3, ERK, MEK and p70 S6 kinase. Among the roles these signaling proteins play in inflammation are mediation of TNFα action, chemokine function, and proliferation of T-cells. Many other cytokines, chemokines and inflammatory molecules similarly require one or more of these molecules to be activated to mediate their effects.
We have developed *SureFire*™ cell based assays for each of these targets, allowing the high throughput, homogeneous detection of activation of each endogenous cellular enzyme, so permitting large scale library screening of these targets using automated liquid handling systems. The *SureFire*™ system utilizes AlphaScreen® (PerkinElmer) and provides a highly sensitive screening platform that can be easily adapted to existing systems and robotics. Examples of screening to be presented will include the analysis of effect of TNFa receptor antagonists, utilizing p38 MAP kinase activation as a readout to accurately indicate inhibitor effectiveness.

p38 MAP Kinase

U937 cells grown in RPMI with 10% serum were centrifuged and resuspended in RPMI with 0.1% BSA without serum at $1x10^7$ cells per ml. After 90 min, cells were stimulated with TNFα for 20min or left unstimulated. Cells were then lysed by the addition to the medium of a 1/5th volume of 5X Lysis buffer. To a sample of this lysate was added a 1/5th volume of Activation Buffer, and 6ul transferred to a ProxiPlate™. To this lysate was added 6ul of Reaction Buffer containing AlphaScreen® beads (1:60 v/v), and the plate was incubated in the dark for 2 hours and then read on a EnVision Alpha™. Results are the Mean+/-SEm of 3 replicates.

Phospho- p38 MAP Kinase

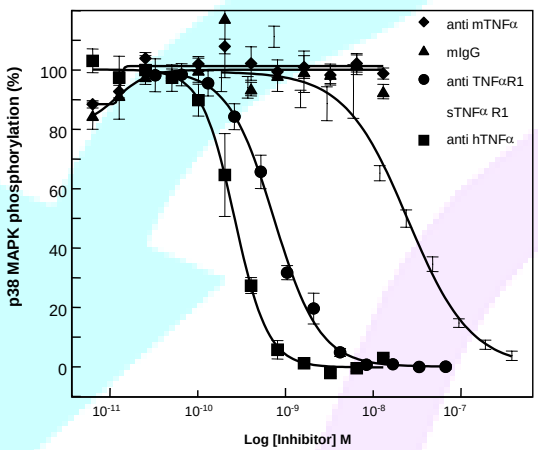


Ranking TNFα signalling inhibitors using p38 MAPK phosphorylation

We have shown that activation of p38 MAPK can be used as a sensitive readout of the level of activation of the TNFα receptor. Using the *SureFire*™ p38 MAPK phosphorylation assay, this provides a high throughput system to analyse the status of receptor activation in a cellular context. This is a significant increase in the level of information one receives from receptor binding studies, as such experiments will not discriminate between effective and non-effective binding of potential receptor antagonists.

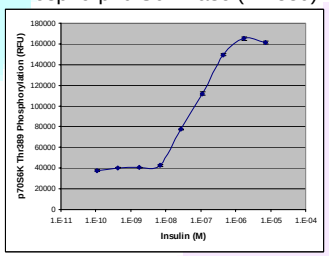
Either the soluble TNFα r1, or antibodies recognising hTNFα, mTNFα and the TNFα R1 were preincubated with U937 cells for 1 h prior to stimulation with TNFα. Cell lysates were subsequently analysed for phosphorylated p38 MAPK as an indicator of receptor signalling. The results suggest antibodies against either TNFα or TNFα R1 were far more potent inhibitors of receptor signalling than the soluble receptor. As expected, neither the antibody rasied against mTNFα, or control mouse IgG, had any effect on receptor signalling.

TNFα Antagonist screening

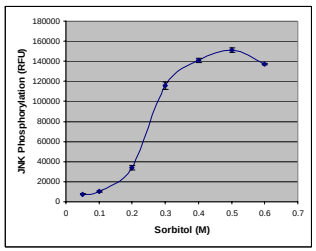


Screening of other signal transduction pathways related to inflammation

Phospho-p70 S6 kinase (Thr389)

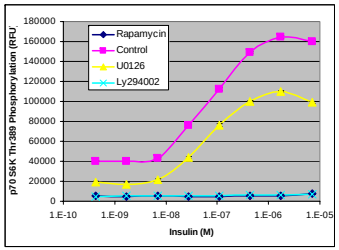


Phospho-JNK



Screening for inhibitors of the PI 3-Kinase / AKT / mTOR pathway

MCF-7 cells were pretreated with no inhibitor (control), rapamycin, LY294002 or U0126 to block mTOR, PI 3-kinase or ERK activation, respectively, and then cells stimulated with insulin. The level of activation of p70 S6 kinase (Thr389) provides a measure of inhibition of the AKT/mTOR pathway through this measurement of the specific phosphorylation site of p70 S6K mediated through mTOR. Further delineation of the site of inhibition could be obtained by screening using the phospho-AKT *SureFire*™ kit.



CONCLUSIONS

The *SureFire*™ assays of phosphorylation of cellular signalling proteins provide a new tool for the rapid detection of bioactive molecules using a biologically relevant system, that of living cells. As the technology is sensitive enough to detect phosphorylation of endogenous cellular proteins, activation of these pathways by endogenous or cloned receptors can be carried out. Therefore, screening of both receptor modulators, such as monoclonal antibodies, as well as small molecule inhbitors of signal transduction pathways can be carried out. Due to the homogeneneous nature of the assays, which utilizes AlphaScreen® technology, HTS programs can be carried out using these assays using standard liquid handling robotics. Current assays allow the assessment of the major MAP kinases, the PI 3-kinase pathway activated protein kinases AKT and p70 S6K, and of Stat-3.
This assay portfolio will be added to in the future to provide an even broader spectrum of targets for the discovery of pharmaceutical agents and for basic research applications.

Contact Information

The *SureFire*™ Cellular Kinase assay kits are formulated for the HTS marketplace for screening large sample numbers and robotic operation, as well as the research laboratory. Further information about kits, prices and protocols can be obtained from the TGR BioSciences Pty Ltd and PerkinElmer Customer Service centres:

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